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Multiplex polymerase chain reaction for identification of bovine mastitis associated pathogens

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Abstract
The present study was focused on the development of a sensitive multiplex polymerase chain reaction (mPCR) for rapid detection of mastitis associated pathogens directly from milk samples. A total of 40 mastitic milk samples were processed for isolation and identification of pathogens which were mentioned below by conventional method and compared it with mPCR. The prevalence of bacterial isolates in conventional detection were found to be 20 (50%) *Staphylococcus aureus,* 10 (25%), *Streptococcus agalactiae,* 9 (22.5%) *Klebsiella pneumoniae and* 8 (20%) *Escherichia coli,* respectively. In mPCR all culture-positive samples were detected for the corresponding bacteria but additional numbers of (i.e. 5 *S. aureus,* 7 *S. agalactiae,* 2 *E.coli,* and 4 *K. Pneumoniae*) isolates were also detected by mPCR which were culture-negative. The target sequence for primer designing were the different gene i.e. *vicK* gene (*S. aureus*), *atr g*ene (*S. agalactiae), barA* gene (*K. pneumoniae*) and *uidA* gene (*E. coli).* It was concluded that, *Staphylococcus aureus* appeared as the most prevalent organism responsible for causing mastitis in cattle. Furthur, the results suggested that mPCR was significantly more sensitive than culture detection. A multiplex PCR could be used as an efficient tool for detecting pathogens of mastitis with high accuracy, sensitivity and in a short period of time.

Keywords: Bovine mastitis, bacteria, conventional method, diagnosis, multiplex PCR

Introduction
Bovine mastitis is devastating disease affecting both human and animal health associated with economic losses due to high morbidity, reduced milk production, discarded milk, increased antimicrobial resistance of the organisms in animals along with cost of veterinary treatment (Awale *et al*., 2012) [3]. Mastitis is a multi-etiological complex disease, characterized by inflammation of the parenchyma of mammary glands and changes in physical, chemical, bacteriological characteristics of milk, pathological transformation in glandular tissues (Radostits *et al*., 2000) [21]. Though various wide spectrums of pathogens have been identified as causative agents of mastitis but some major bacterial species are responsible i.e. *S. aureus*, *S. agalactiae, K. pneumoniae* and *E. coli* (Riffon *et al*., 2001 and Bannerman *et al*., 2003) [23, 5]. In most clinical laboratories, identification methods are based on the microbiological culture of milk, antimicrobial sensitivities and biochemical tests on the bacterial isolates. However, there are several disadvantages associated with microbiological culture i.e. it is limited by the dynamic nature of infections. Milk culture may yield no bacteria from truly sub-clinically infected glands due to the presence of very low numbers of pathogens when samples are collected. Negative cultures may also result due to various factors such as residual therapeutic antibiotics or presence of leukocytes with high Somatic Cell Count in milk. Moreover, the microbiological culture of milk is time-consuming. Species identification by standard biochemical methods requires more than 48 h to complete. Due to the limitations of cultural methods, DNA sequence-based molecular methods have been gaining much attention with an approach for species identification (Cremonesi *et al.*, 2009; Zadoks and Watts, 2009) [7, 29] and genome sequences of these pathogens are utilized to develop rapid, sensitive and specific nucleic acid-based testing methods such as mPCR assay which can be used for detection of more than one pathogen in single PCR reaction enhancing early detection of mastitis. The identification of bacteria at the species level in mPCR is based on the amplification of a conserved house-keeping gene sequence, which is highly conserved within the species, but variable between species.

These regions are amplified by primers designed from such conserved gene for selected pathogen like *vicK* gene for *S. aureus* (Liu *et al.*, 2007) [16], *atr* gene for *S. agalactiae* (Munari *et al.*, 2012) [18], *BarA* gene for *K. pneumonia* and *uidA* gene for *E. coli* (Martins *et al.*, 1993) [17] respectively.
The aim of present study was to develop a rapid and early detection method which focused on the simultaneous detection of the pathogens of mastitis directly from mastitic milk i.e. *S. aureus*, *S. agalactiae*, *K. pneumonia* and *E.coli* through multiplex PCR.

Material and Methods

1. Source of milk samples

A total of forty milk samples were collected from cattle showing symptoms of (clinical and subclinical) mastitis confirmed by the California Mastitis Test. The samples were collected from the Veterinary Clinical Complex and dairy farms in and around the Bikaner city, Rajasthan, India. These samples were collected before milking using standard procedures described by the National Mastitis Council (Oliver *et al.*, 2004) [19].

2. Isolation and identification of bacteria

a) Culture characteristics

The milk samples were subjected to aerobic cultivation. Each milk sample was inoculated in nutrient broth overnight and then streaked on nutrient agar followed by overnight incubation at 37 ºC. Bacterial colonies were closely observed for their morphology, colour and consistency. Gram's staining was used as primary identification test and these presumptive colonies were streaked on Mannitol salt agar (MSA), Edward's medium agar, MacConkey agar and Eosin methylene blue agar (EMB) plates, for isolation of *S. aureus*, *S.agalactiae*, *K. pneumoniae* and *E.* coli, respectively. These agar plates were incubated for 24 hours at 37 °C under aerobic condition. The growth was examined for the colonial morphology, fermentation, pigment production and other characteristics.

b) Biochemical characteristics

Cultures were subjected to different primary biochemical tests *viz*. Catalase test, Oxidase test, Oxidation-Fermentation test and motility test for all isolates. Isolates identified presumptively as *S. aureus* were confirmed by carried out tube coagulase test using human plasma, mannitol fermentation test, DNase test, haemolytic properties and fermentation of different sugars whereas *S. agalactiae* subjected for growth on Edward's medium, Christie, Atkins, and Munch-Petersen (CAMP) test, hydrolysis of esculin, Arginine hydrolysis test, haemolytic properties on blood agar and Voges-Proskauer test. Gram-negative isolates such as *K. pneumoniae* and *E. coli* were evaluated by their biochemical reactions on the following: Growth on Mac-Conkey agar, EMB agar, IMViC tests, growth on Triple Sugar Iron agar, Sugar fermentation tests, Urease test, Nitrate reduction test, and utilization of lysine, etc.

3) DNA extraction directly from mastitis milk samples

All the samples were exposed to extraction of DNA directly from mastitis milk. DNA extraction directly from milk was carried out using the method Phenol-Chloroform Extraction described by Phueketes *et al*. (2001) [20] with certain alterations. The integrity of DNA was checked by gel electrophoresis used 1.5% agarose whereas, DNA quantification was carried out by spectrophotometric measurements. The quantified DNA was diluted to a final concentration of this 25 ng/µl in Tris-EDTA (TE) buffer.

4) Desining of oligonucleotide primers

PCR primers were designed from using highly conserved housekeeping genes included *vicK* gene for *S. aureus* (Liu *et al.*, 2007) [16], *atr* gene for *S. agalactiae* (Munari *et al.*, 2012) [18], *BarA* gene for *K. pneumoniae* and *uidA* gene for *E. coli* (Martins *et al.*, 1993) [17]. The sequence of primer, target gene, and product size are summarized in Table 1. All primers had similar annealing temperatures, which is essential for multiplex PCR production. These primers were synthesized by Primer3 software. The Insilico PCR tool was used to predict primer location, orientation, length of each amplicon and ability of primers to amplify particular species (Specificity and sensitivity). Then MFEprimer-2.0 software was used for checking possible dimer formation in between inter and intra primer in multiplex PCR. (Qu *et al*., 2012) [21]. To establish a combination of sets of primers for multiplex PCR, similar Tm values was predicted by the Oligo analyzer tool. All these primers were resuspended to a final concentration of 100 µM in deionized autoclaved water.

Table 1: Primers used for amplification of *Staphylococcus aureus*, *Streptococcus agalactiae* and *Escherichia coli* DNA in bovine milk samples by multiplex-PCR

Agent	Target	Primers	Sequences (5'-3')	PCR product size(bp)	Annealing temp.
S. aureus	*Vick* gene	js SA F	CAGACCGTCGTGGACGTATT	264	58°C
		js SA R	TCACGTCATGTAACACAGCGA		
S. agalactiae	*Atr* gene	jsStrAg F	CCCTTCTGGCTCTGGTAAGTC	304	
		jsStrAg R	TGCTGGATAAGCATTAGCCTTCT		
K.pneumoniae	*barA* gene	js KN F	GATGGGCGGGGATATTTCGT	555	
		js KN R	TTCAGGTTAGCCGGGTTGTC		
E.coli	*uidA* gene	js*EC F*	TACCGACGAAAACGGCAAGA	119	
		js*EC R*	CGGTGATATCGTCCACCCAG		

5) Multiplex PCR amplification and analysis

The reaction for multiplex PCR carried out in a final volume of 25 µl consisted of 5.0 µl 5X Go Taq® Flexi buffer, 3.0 µl MgCl2(25mM), 4µl primer-F(10 pM/µl) (1µl of individual primer), 4µl primer-R (10 pM/µl) (1µl of individual primer), 1 µl dNTP (25mM each), 0.25 µlTaq DNA polymerase (5 U/µl), 3 µl template DNA (30ng/µl) and 4.75 µl nucleus free water. The pre denaturation at 96°C for 5 min was applied. A total of 35 PCR cycles were run under the following conditions: denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min., final extension at 72°C for 10 min, and PCR products were stored at 4°C. Amplification product were electrophoresed at 100V/cm for 1 hour in 1.5% agarose gel prepared in 1.0XTBE buffer containing 0.5 ng/ml ethidium bromide and visualized by ultraviolet light trans illumination (UVP Gel Doc Bio imaging System).

Results and Discussion

Conventional Microbiological Analysis

Conventional determination was conducted for the above mentioned bacterial pathogens on the basis of cultural and biochemical identification. Milk samples collected from mastitis cases and examined bacteriologically, out of 40 samples, 27 (67.5%) was yielded at least one of the selected target pathogens. A total 47 bacterial isolates have been identified; *S. aureus, S. agalactiae, K. pneumoniae, and E. coli* were recovered in percentage of 50, 25, 22.5 and 20 respectively. It was concluded that, *Staphylococcus aureus* appeared as the most prevalent organism responsible for causing mastitis in cattle. Kurjogi *et al.,* (2011) [14] also reported that major pathogens isolated from the milk samples were *S. aureus and S.agalactiae*. The prevalence of *Staphylococcus* species might be due to the incomplete milking, painful lesions or any wounds on the outer surface of the udder.

Staphylococcus aureus:- Preliminary biochemical characterization of all 20 isolates of *S. aureus* revealed characteristic growth pattern on mannitol salt agar (MSA) fermenting mannitol and produced characteristics golden yellow colonies, and showed grapes like appearance under microscope after Gram staining (figure 1).

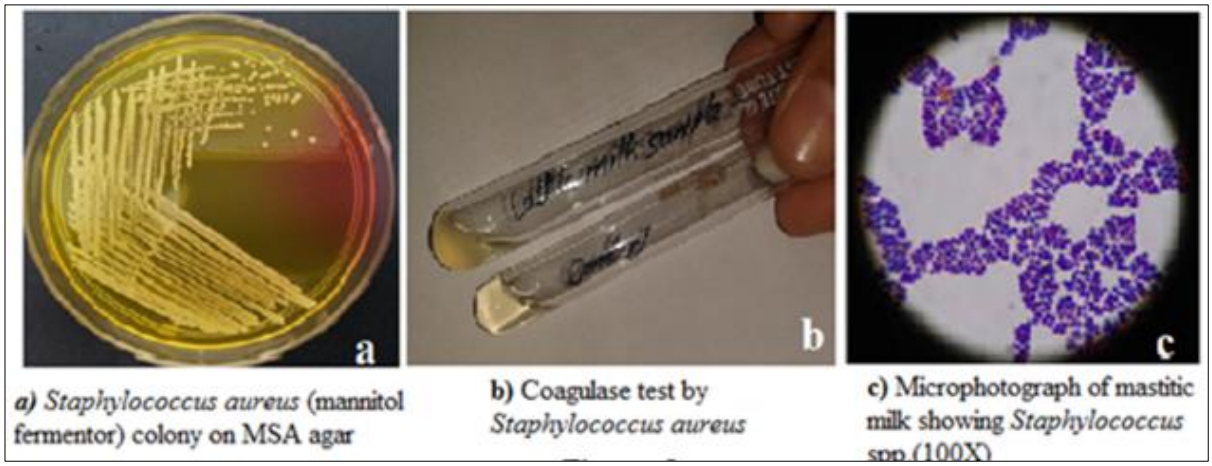


Fig 1.

The 85% isolates also produced hemolysis on blood agar (alpha and beta hemolysis in 4 and 13 isolates, respectively) (Table 2). The results of the present study are in complete agreement with that of Upadhyay & Kataria, (2010) [28] and Gangwal *et al.,* (2016) [9] who recorded the haemolytic properties of *S. aureus* obtained from milk samples of cattle and goats. Total of 70% and 80% isolates were recovered for DNAse test, coagulase production (Figure.1) respectively and fermented different sugars (Table 2). Similar finding was reported by Kateete *et al*., (2010) [12] who detected that 75% and 91% isolates of *S. aureus* were DNAase positive, coagulase positive in that order. Yadav *et al.,* (2015) [29] who obtained 32 *S. aureus* isolates from cattle and buffalo and they all showed a strong coagulase reaction.

Table 2: Biochemical characterization of *Staphylococcus aureus*

Test	Positive	
	Number	percentage
DNAse test	14/20	70%
Haemolysis	17/20	85%
	4 α	13 β
Coagulase	16/20	80%
Mannitol	20/20	100%
Trehalose	19/20	95%
Lactose	20/20	100%
Maltose	18/20	90%
Sucrose	20/20	100%
Arabinose	2/20	10%
Raffinose	1/20	5%

Streptococcus agalactiae:- *Streptococcus agalactiae* prevalence were obtained in 25% samples. The result, obtained in this study was accordance with the earlier workers from India, who reported incidence of *Streptococcus agalactiae* in mastitic milk was 14.01% (Kuler, 2006) [13]. These isolates were showed dewdrop like colonies on Edward's medium, revealed utilization of arginine, positive for VP test, absence of hydrolysis of esculin and fermented different sugars (Table 3). Daignault *et al.,* (2003) [8] selected total of 295 *S. agalactiae* isolates were showed positive CAMP reaction, not to hydrolysed esculin, 95% of the isolates produced a beta-hemolysis and 5% were non-hemolytic. On Blood agar plates all isolates produced haemolysis. Our observations based on haemolysis are supported by the report of Bhagat *et al*., (2015) [6]. *Streptococcus agalactiae* was also studied for sugar fermentation activity *viz*. Glucose, sucrose, lactose, and utilization of arginine. This *Streptococcus agalactiae* isolate did not ferment arabinose, sorbitol, mannitol and raffinose (Table no.3). Our result was comparable to that of Al-kuzaay *et al.,* (2013) [1] sugar fermentation results.

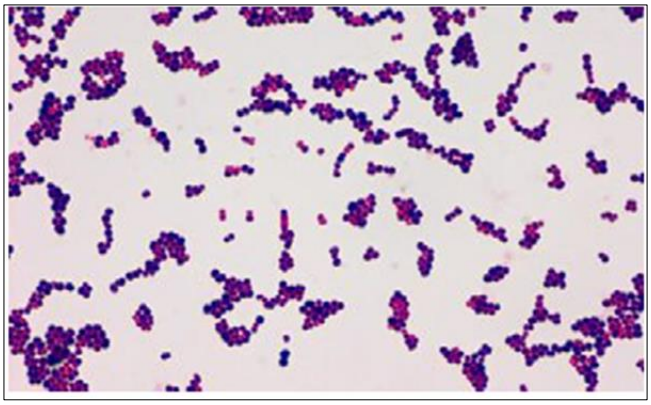


Fig 2: Microphotograph of mastitic milk showing *Streptococcus spp.* (100X)

Table 3: Biochemical characterization of *S.agalactiae*

Biochemical characters	Positive	
	Number	percentage
Growth on Edward's Media	10/10	100%
Hemolysis	10/10	100%
VP test	8/10	80.00
Esculin Hydrolysis	1/10	10.0%
Arginine Utilizatation Test	9/10	90.0%
Glucose	10/10	100%
Lactose	6/10	60.0%
Arabinose	0/10	0%
Sorbitol	0/10	0%
Sucrose	10/10	100%
Mannitol	1/10	10.0%
Raffinose	0/10	0%

***Klebsiella pneumoniae*:** All 9 isolates of *K. pneumoniae* on preliminary identification showed biochemical characterization i.e. mucoid colonies on MacConkey agar with string formation, gram–ve rod shape in staining (Figure 3). These isolates revealed characteristic IMViC pattern (- - + +.), positive urease activity (Figure. 5) and growth on TSI (Y/Y/-) agar (Table.4).

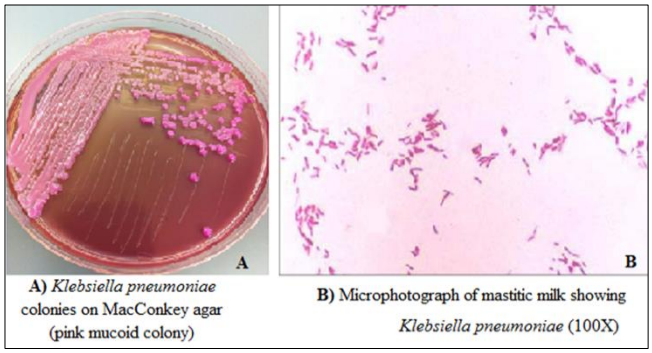


Fig 3.

Our result was similar with Alves *et al.*, (2006) [2] who reported that a total of 16 conventional and 4 supplementary tests were used to evaluate isolates identified as *Klebsiella* sp., out of these, 84% isolates were identified as *Klebsiella pneumoniae* that were negative for indole production, citrate positive, non-motile typical gram-negative bacilli, unable to assimilate histamine and d-melezitose or to grow at 10°C.

Table 4: Biochemical characterization of *K. pneumoniae*

Test	Positive	
	No	%
Growth on Mac conkey	9/9	100%
Indole test	0/9	0%
MR Test	5/9	55.5%
V-P Test	9/9	100%
Citrate utilization test	9/9	100%
Growth on TSI	7/9	77.7%
Urease Test	9/9	100%

***E. coli*:** All 8 isolates of *E. coli* on preliminary biochemical characterization revealed growth on MacConkey agar and produced metallic sheen on EMB agar (Figure 4). Similar finding was recorded by Leininger *et al.*, (2001) [15].

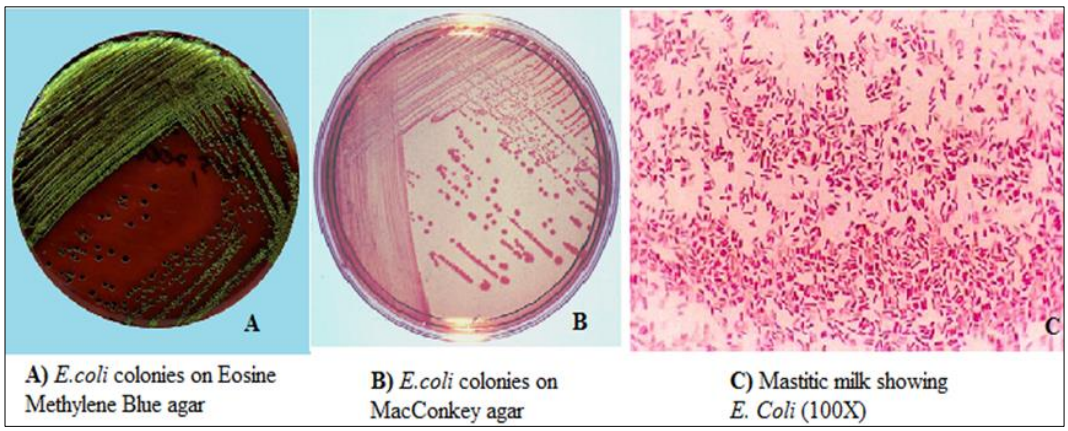


Fig 4.

This appearance of metallic sheen on culture was due to the formation of eosinate from eosine. They all isolates showed characteristic IMViC (+ + −) pattern, and fermented different sugars (Table no. 5). They were also reported as Urease negative (Figure. 5) means not utilize urea and it is completely agreed with Rangel *et al.*, (2009) [23] finding. These findings nearly supported the Haque *et al.*, (2014) [11] and Gangwal *et al.*, (2016) [9] in which isolates of *E.coli* revealed positive reaction in MR test and Indole test but negative reaction in VP test and by fermentation of sugar they produced acid and gas.

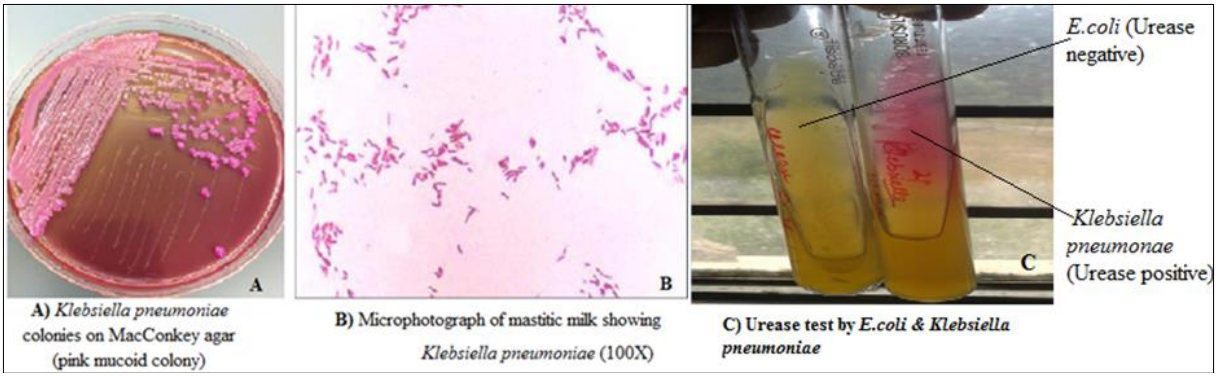
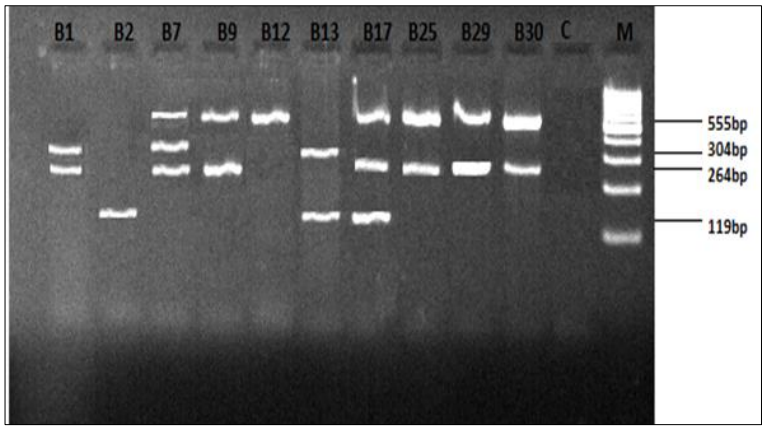


Fig 5.

Table 5: Biochemical characterization of *E. coli*

Test	Positive	
	Number	percentage
Growth on EMB	8/8	100%
Urease Test	0/8	0%
Indole test	8/8	100%
MR Test	8/8	100%
V-P Test	0/8	0%
Citrate utilization test	0/8	0%
ONPG	7/8	87.5%
Lysine utilization	8/8	100%
Nitrate reduction	8/8	100%
Sucrose	6/8	75%
Glucose	8/8	100%
Lactose	7/8	87.5%

Molecular diagnosis of common bacterial pathogens directly from bovine mastitic milk-The present study revealed that multiplex PCR method was very rapid and early detection tool to determine bacterial etiology of mastitis milk samples simultaneously at a time in single reaction with lesser reagents than simplex PCR, which was similar to that of earlier reports (Phuektes *et al*., 2001; Gillespie *et al.*, 2005) [20, 10]. DNA was isolated from 40 milk samples by method described by Phuektes *et al.*, (2001) [20] with slight modifications. Results obtained from multiplexing showed simultaneous detection of *S. aureus* 25 (62.5%), *S. agalactiae* 17 (42.5%), *K. pneumoniae* 13 (32.5%), and *E.coli* 10 (25%). The respective pathogens were visible under agrose gel electrophoresis where amplified DNA fragments showed species-specific amplicons of size 264bp, 304bp, 555bp, 119bp corresponding to *S.aureus, S.agalactiae, K. pneumoniae* and *E.coli* (Figure.6). However, Shome *et al.*, (2011) [25] were able to detect 10 bacterial strains simultaneously by mPCR. Azevedo *et al.*, (2016) [4] detected Coagulase Negative Staphylococcus, *E. coli*, *S. aureus* and other coliform bacteria (*Klebsiella oxytoca*, *Klebsiella pneumonae* and *Serratia marcescens*) in 100, 75, 59, and 35 % of Bulk Tank Milk, respectively by mPCR.



M- marker 100bp, C- control, B- sample no.
119bp- *E.coli*, 264bp- *S.aureus*, 304bp -*S.agalactiae*, 555bp -*K. pneumoniae*

Fig 5: Sensitivity of multiplex PCR in direct detection of pathogens directly from milk sample

Differential Sensitivity of conventional methods and Multiplex PCR on Milk Samples Upon comparison, the multiplex PCR could detect more species at a time, from the DNA which was isolated directly from milk samples than that of conventional methods. In multiplexing all culture positive samples were detected positive for the corresponding bacteria (*S. aureus*, *S. agalactiae*, *K. pneumoniae*, and *E. coli*) but milk samples that were negative for these pathogens by culture method, found to be positive by multiplex PCR. The comparative detection of corresponding bacteria is depicted in Table 6 and Figure no. 6. In comparison of mPCR with isolation of the pathogens, the mPCR detected more isolates (*n*= 65) as compaired to phenotypic determination (*n*=47). The additional isolates were also detected by mPCR in 8 samples which were culture-negative for the corresponding species. Five samples were negative for both cultural isolations as well as from mPCR detection. A higher detection ranging from 2 to 7 was observed in mPCR when compared to culture for selected species. The probable reasons for the detection of additional organisms by mPCR might be due to non viability of the particular bacteria, which did not create interference in detection by PCR technology. Besides, the discrepancy in the number of different bacteria present in milk may be due to result in growth of the dominant species

inhibiting the growth of minor species. In a similar way Taponen *et al.*, (2009) [27] and Shome *et al.*, (2011) [25] reported a large proportion of positive samples via molecular methods from culture-negative milk samples in conventional bacteriology.

Table 6: Comparison of detection of target pathogens in mastitic milk samples by multiplex PCR and culture

Organism	Genotypic determination Number of isolates (%)	Phenotypic determination Number of isolates (%)
Staphylococcus aureus	25 (62.5%)	20 (50%)
Streptococcus agalactiae	17 (42.5%)	10 (25%)
Klebsiella pneumoniae	13 (32.5%)	9 (22.5%)
E.coli	10 (25%)	8 (20%)
Total isolates	65	47
Positive sample	35	27
Negative sample	5	13
Sensitivity	more	less

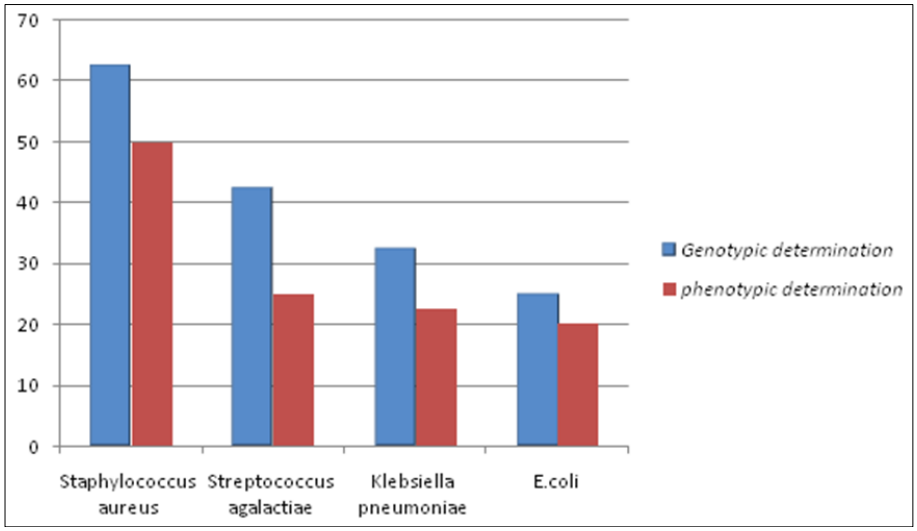


Fig 6: Comparison of detection of target pathogens in mastitic milk samples by multiplex PCR and culture

Conclusion

It can be concluded that DNA isolation and mPCR methods developed in this study are more sensitive and faster than conventional culture, and can be easily applied for detection of *S. aureus*, *S. agalactiae*, *K. Pneumonia* and *E. coli* in milk samples simultaneously. It can therefore be used as alternative to conventional culture method in the routine diagnosis. Early detection of mastitis by mPCR provide correct species identification at the initial stage and reveal exact etiology, furnish appropriate assistance or medical aid for the affected animal. It may, contribute a fruitful role in surveillance programs, prevention, control strategies and in epidemiological investigations. Also this study would be basis of further studies that aim to optimize and perform new processes for examining other bacterial agents that cause mastitis. In conclusion, the developed mPCR assay can be used for rapid, sensitive, specific and reliable identification of the major mastitis pathogens from milk.

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